Detection of anaerobic carbon monoxide-oxidizing thermophiles in hydrothermal environments.

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CITATION:

Yoneda, Yasuko ...[et al]. Detection of anaerobic carbon monoxide-oxidizing thermophiles in hydrothermal environments.. FEMS microbiology ecology 2015, 91(9): fiv093.

ISSUE DATE:

2015-09

URL:

http://hdl.handle.net/2433/207705

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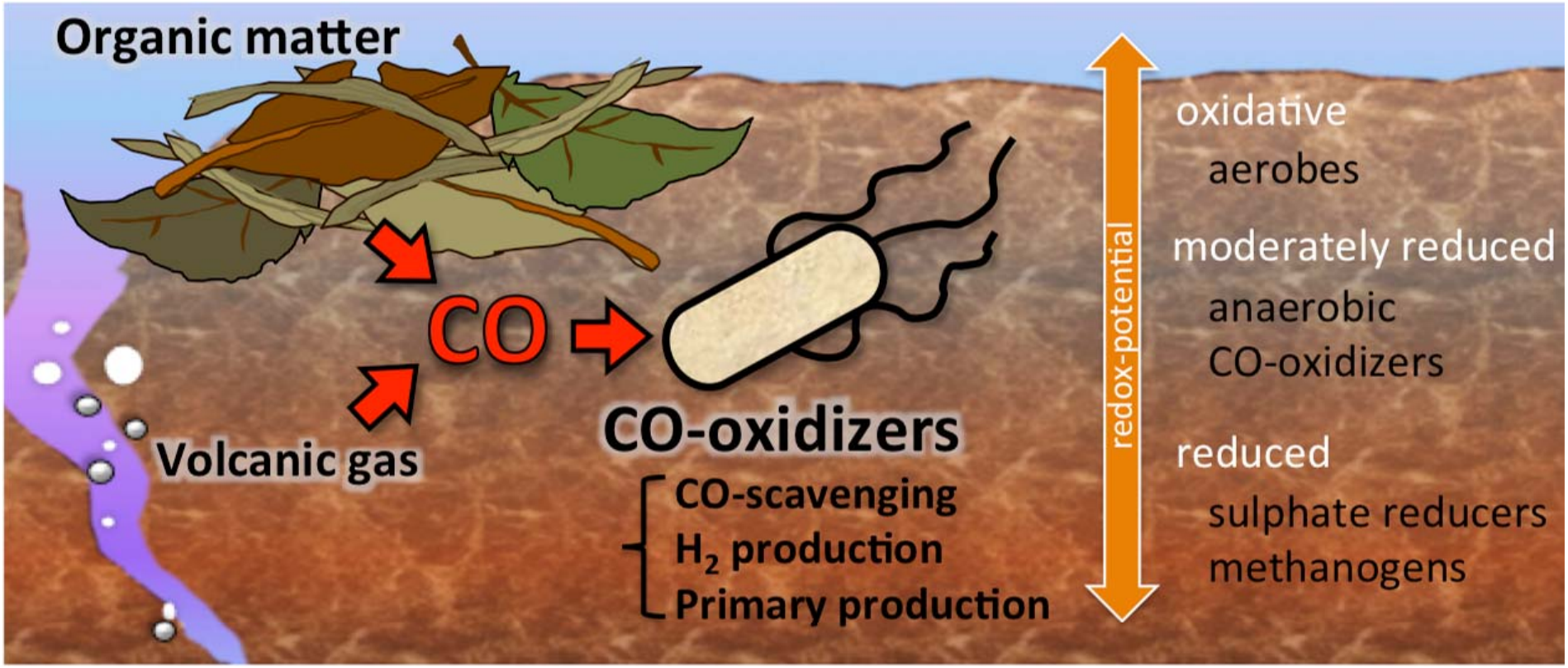
Abstract

Carboxydotrophic anaerobic thermophiles have been isolated from various hydrothermal environments and are considered to be important carbon monoxide (CO) scavengers or primary producers. However, the ecological factors that influence the distribution, abundance and CO-oxidizing activities of these bacteria are poorly understood. A previous study detected the carboxydotrophic bacteria *Carboxydothermus* spp. in a hot spring sample and found that they constituted up to 10% of the total bacterial cells. In this study, we investigated environmental features, potential microbial CO-oxidation activities and the abundance of *Carboxydothermus* spp. in various hot springs to determine environmental factors that affect CO-oxidizers and to see whether *Carboxydothermus* spp. are common in those environments. We detected potential microbial CO-oxidation activities in samples that showed relatively high values of total organic carbon (TOC), total nitrogen (TN), oxidation–reduction potential (ORP) and soil-water content. The abundance of *Carboxydothermus* spp. did not correlate with the presence of potential microbial CO-oxidation activities; however, *Carboxydothermus* spp. were detected in a wide range of environments, suggesting that these bacteria are widely distributed in spite of the relatively low population size. This study implies thermophilic CO-oxidizers occur in a wide range of environments and oxidize CO in somewhat oxidative environments rich in organic matter.



Graphical Abstract Figure

One-sentence summary

Carbon-monoxide-oxidizing thermophiles are widely distributed in a range of environments, and high potential microbial CO-oxidation activity is associated with the levels of organic matter, oxidation–reduction potential and soil-water content of environments.

Keywords

carboxydotroph, carbon monoxide, carbon monoxide dehydrogenase, thermophile, hot spring

Introduction

It is believed that approximately 2,500–2,600 Tg of carbon monoxide (CO) is produced annually and emitted to environments via the combustion of fossil fuels, oxidation or photochemical degradation of organic compounds, volcanic gas, and metabolism in animals, plants and microbes (King & Weber, 2007). CO concentrations in environments usually occur in trace amounts; e.g., 2–15 nM in seawater (Moran & Miller, 2007), 20–33 nM in hot springs (Kochetkova *et al*., 2011) and approximately 92 ppb global mean mole in the atmosphere (WMO WDCGG, 2014). There is generally equilibrium between CO production and consumption; approximately 90% of atmospheric CO is consumed by reaction with hydroxyl radicals, and CO-oxidizing microbes in soil are considered to be a CO sink accounting for 10% of the total CO consumption (King, 2007).

Studies on microbiological CO consumption have been conducted under aerobic conditions using soil, freshwater and seawater (Conrad & Seiler, 1980; Conrad & Seiler, 1982). Aerobic CO-oxidizers utilize molybdenum-containing carbon monoxide dehydrogenases (Mo-CODHs), which can be detected widely in environments such as soils (plant root), sediments, volcanic deposits and the ocean floor (King, 2003; King, 2007; Dunfield & King, 2004; Martin-Cuadrado *et al*., 2009). In anaerobes, CO oxidation is catalysed by nickel-containing CO dehydrogenases (Ni-CODHs) (Ragsdale, 2004). Ni-CODHs can be divided into the following two major groups: the monofunctional CODH group and the bifunctional CODH/acetyl CoA synthase (ACS) group. Bifunctional CODH/ACSs comprise CODH subdomains and

ACS subdomains and are known to be key enzymes in the Wood–Ljungdahl pathway (reductive acetyl CoA pathway) (Ragsdale, 2004). The CODH subdomains reduce CO_2 to CO, after which the ACS subdomains incorporate CO as a carboxyl group of acetyl-CoA. Monofunctional CODHs catalyse oxidation of CO to CO_2, thereby deriving low potential electrons whose energy can be converted to the transmembrane potential and ATP when they are transferred to some oxidants (Techtmann *et al*., 2009). In a previous study, monofunctional and bifunctional CODH genes were predicted from their genomic context. Monofunctional Ni-CODH genes are widely found in the prokaryotic genomes of hydrogenogens, methanogens, sulphate reducers, acetogens and gut microbes in animals (Techtmann *et al*., 2012). CODH genes are found in diverse microbes in various environments; however, only a few studies have reported anaerobic microbial CO oxidation in environmental samples from soil, wetland and hot springs (Conrad & Seiler, 1980; Rich & King, 1999; King, 2007; Kochetkova *et al*., 2011), and the environmental and physiological significance of anaerobic CO-oxidizers is less understood.

The thermophilic bacterial genus *Carboxydothermus* is one of the most studied CO-oxidizing anaerobes. Five species of the genus *Carboxydothermus* have been isolated from hot springs and described (Yoneda *et al*., 2012; Sokolova *et al*., 2013). Four of the species produce hydrogen via CO-oxidization under 100% CO atmospheric conditions. Because CO is a toxic gas and hydrogen is an important energy source for many microbes in an anoxic environment, CO-oxidizers in the environment are assumed to be important ‘CO scavengers’ or primary producers (Sokolova *et al*.,

2009; Techtmann *et al*., 2009).

The genome of *C. hydrogenoformans* has four genes encoding monofunctional CODH (*cooS-I*, *cooS-II*, *cooS-IV* and *cooS-V*), which have different predicted functions and sequences (Wu *et al*., 2005). Previously, the quantitative detection of *Caboxydothermus* spp. by targeting the CODH-II gene (*cooS-II*) using real-time PCR showed that these microorganisms can constitute up to approximately 10% of the total bacterial cells in acidic hot spring sediment where *Carboxydothermus pertinax* was previously isolated (Yoneda *et al*., 2013a). Considering that the optimal growth pH of *Carboxydothermus* spp. is moderately acidic or neutral, the abundance of these bacteria is intriguingly high. This led us to further question whether *Carboxydothermus* spp. are common in other hot springs, where the environment is more suitable for the growth of this genus, and are responsible for CO-oxidation *in situ*. The physiological group of carboxydotrophic hydrogenogens has been isolated from various environments, such as hot springs, deep-sea hydrothermal vents, lake sediments and bioreactors (Sokolova & Lebedinsky, 2013). Unfortunately, the range of environments that offer a niche for these microorganisms is still unknown. The aim of the current study was to determine the answer to the above question. In the current study, we measured potential microbial CO-oxidation activities under anoxic conditions and the abundance of *Carboxydothermus* spp. in various hot spring samples.

Materials and methods

Sample collection

Sample sediments were collected from hot springs located in the Kagoshima Pref. (southern Kyushu Island) and Shizuoka Pref., Japan (Fig. 1). The hot springs were Unagi-onsen in the Kagoshima Pref. and Atagawa-onsen (Benzainoyu and Izu Atagawaso), Shimogamo-onsen (Jiunji temple), Mine-onsen (Mine Onsen Fountain Park), Yatsu-onsen (Yakushinoyu) and Yugawara-onsen (Daikan-Soh) in Shizuoka Pref. As references, samples were collected from the Unagi Lake near Unagi-onsen, a water drain in Shimogamo-onsen and the Yamagawa coastal hydrothermal field that had high salinity (Table 1). The Unagi Lake and a water drain near Shimogamo-onsen were non-hydrothermal environments. The surface layer (above a 3.0 cm depth from the water-sediment interface) of the sediments was sampled using a plastic core tube (7.0 cm diameter) or using a metal dipper. Samples were thoroughly mixed using metal spoons and then subsampled into plastic tubes. For measurement of potential microbial CO oxidation activities, samples were placed in a plastic bag with AneroPouch–Anaero (Mitsubishi Gas Chemical, Tokyo, Japan) and sealed immediately. All sample tubes were packed with ice and transported to the laboratory. Potential microbial CO oxidation was measured as soon as possible after return to the laboratory. Samples for DNA extraction were stored at −80 °C until use. The other samples were refrigerated at 4 °C until use.

Physiochemical analyses of the environment

The temperature, pH and oxidation–reduction potential (ORP) of the samples were measured before sample collection using a portable meter HM-31P (DKK-TOA, Tokyo,

Japan). Soil-water content was calculated on the basis of the weight of the sediments before and after overnight oven-drying at 105 °C. Pore water was extracted by filtration using filter paper (Advantec quantitative ashless filter paper grade no. 5C, Toyo Roshi Kaisha, Tokyo). The total organic carbon (TOC) and total nitrogen (TN) contents of pore water were analysed using TOC-L (Shimadzu, Kyoto, Japan) equipped with a TN meter unit, TNM-L (Shimadzu). The salinity of pore water was measured using a digital salinity probe SS-31A (Sekisui Chemical, Osaka, Japan) in the laboratory at room temperature.

A principal component analysis (PCA) was performed using R 3.0.2 (R Development Core Team, 2008) to compare the environmental features of the samples.

Measurement of potential microbial CO oxidation

Samples were manipulated in an anaerobic glove box (Coy Laboratory Products, Grass Lake, MI, USA) under a 5% H_2/ 95% N_2 atmosphere. Approximately 5.0 mL of sediment was placed in each glass vial (63.8 mL), which was then sealed with a rubber stopper. Glutaraldehyde solution (25% v/v), which is reported to effectively inhibit themophilic microbial CO-oxidation (Slepova *et al.*, 2007), was added to the control samples (5% v/v at final concentration). All of the following manipulations were conducted outside of the anaerobic glove box. Glass vials were vigorously vortexed and pre-incubated at experimental temperatures for 1 h. The experimental temperature for each sample was set as close to the temperature of its origin as possible. Five mL of pure CO gas (CO PURE, Sumitomo Seika Chemicals, Osaka, Japan) was introduced in

each glass vial using a syringe (under atmospheric pressure at room temperature). From the start time at CO injection, 1.0 mL of gas phase was subsampled from each vial using a gas-tight syringe at approximately 24 h intervals over 5 days. CO concentrations in subsamples were measured by gas chromatography as previously described (Yoneda *et al*., 2012). Potential microbial CO-oxidation in each sample was calculated based on the difference in the average CO concentrations between the samples and controls. The potential microbial CO-oxidation rate was evaluated by regression analysis and regarded as positive when the slope of microbial CO-oxidation increased over the time course ($p<0.05$). Measurements of CO concentration in samples and controls were performed in triplicate and duplicate, respectively.

DNA extraction and real-time PCR

DNA extraction was conducted using the commercial kit Extrap Soil DNA Kit Plus ver. 2 (NIPPON STEEL and SUMIKIN eco-tech, Tokyo, Japan) following the manufacturer's protocols. Bead-beating steps were carried out using a Beads Crusher μT-12 (Taitec, Koshigawa, Japan) at a speed of 3,200 r/min for 60 s.

Quantitative PCR (qPCR) was performed for the *Carboxydothermus* CODH-II gene (*cooS-II*), the bacterial 16S rRNA gene and the archaeal 16S rRNA gene as previously described (Yoneda *et al*., 2013a). Real-time PCR primers should be designed against conserved regions of closely related strain sequences to cover the target organisms. However, the primer set for *Carboxydothermus cooS-II* cooS2_1442F (5'-TGATGCGTCACGGCTTTATGG-3') and cooS2-1606R

(5'-CTAAAGCTACTGCCCGGGAGT-3') were designed based on the *cooS-II* genes of *C. hydrogenoformans* and *C. pertinax* (Yoneda *et al*., 2013a) because no other sequences closely related to *Carboxydothermus* species were available. No sequence matches to the primers were found in NCBI nr database using BLASTn search. This primer set can also amplify *cooS-II* from the genomic DNA of *Carboxydothermus siderophilus*, but not *Carboxydothermus ferrireducens*. Therefore, the abundance of CODH copy numbers observed in this study might be underestimated. Bacterial and archaeal 16S rRNA gene primer sets were described previously (Einen *et al*., 2008). For *cooS-II*, qPCR was conducted in 4 to 7 replicates. The abundance of bacterial and archaeal 16S rRNA genes was detected in triplicate. Some representative samples of *cooS-II* qPCR products were purified, cloned and sequenced as previously described (Yoneda *et al*., 2013b). The obtained sequences were analysed using the MEGA 6 program (Tamura, 2013).

Results

Sample description

At Unagi-onsen, samples designated UG-20, UG-55 and UG-90 were collected from three points with different temperatures in a hot spring pool. These samples were acidic in the range of pH 2.8-5.3 (Table 1). As a reference, sediments from the Unagi Lake, a freshwater maar lake located approximately 300 m downhill away from the sampling site at Unagi-onsen, were also collected and designated by the label U-Lake. Sample YG-65 was taken from beach sands under hot water flow at Yamagawa coastal

212 hydrothermal field, where general physicochemical and microbial characteristics have
213 been previously studied (Kawaichi *et al*., 2013). The hot water of Yamagawa had a high
214 salinity of up to 2.2% (w/v). Hot springs in the Shizuoka Pref. were neutral to slightly
215 alkaline (pH 7.1–8.4) (Table 1). At Atagawa-onsen, deposits from open-air pools of the
216 hot spring well at Benzainoyu and Izu Atagawaso were designated by the labels BZ-65
217 and AT-90, respectively. At Yakushinoyu in Yatsu-onsen, two samples (samples KS-90
218 and KS-65) of hot spring deposits and mud were collected from open-air hot pools
219 created by continuous hot water inflow. A sample from Mine-onsen designated by the
220 label ME-90 was composed of white sinters scratched out from a pipe that was used to
221 upwell underground hot water. Sediment samples from Shimogamo-onsen were
222 collected from a trench drain of hot spring water in the grounds of the Jiunji Temple
223 (Samples JI-70 and JI-65). Salinity was as high as 0.7% (w/v) in samples JI-70 and
224 JI-65. Sediment samples designated by the label J-Drain were also collected from a
225 water drain that was next to, but not connected to, the hot water drain in the grounds of
226 the Jiunji Temple. Sample DA-80 was collected from a small hot water container owned
227 by Daikan-soh inn in Yugawara-onsen. White and orange-coloured hot spring deposits
228 were collected from the container, which is usually closed with a cover.
229
230 **Potential microbial CO-oxidation and environmental factors**
231 Incubation temperature settings for each sample are shown in Table 2. Potential
232 microbial CO-oxidation was observed in UN-55, UN-90, JI-70, JI-65 and J-Drain (Fig.
233 2). In samples that were positive for potential microbial CO-oxidation activities, ORP,

234 TOC and TN ranged from −49 mV to 487 mV, 14.7 mg/L to 147.1 mg/L and 3.7 mg/L
235 to 10.8 mg/L, respectively (Table 1). The highest amount of potential microbial CO
236 consumption was observed in JI-65, where as much as 63.6% of initial CO was
237 consumed during the experimental period of 5 days (Table 2). Relatively high CO
238 consumptions were also observed in J-Drain (51.2%), UN-55 (46.1%) and JI-70
239 (30.4%). Among the hot spring samples from Unagi-onsen (UN-20, UN-55 and UN-90)
240 and Shimogamo-onsen (JI-65 and JI-70), potential microbial CO-oxidation activities
241 were the highest at temperatures of 55 °C and 65 °C, respectively. These results were
242 expected as most of CO-oxidizing thermophilic isolates from terrestrial hot springs
243 show optimal growth at temperatures of approximately 55–65 °C (Sokolova *et al*.,
244 2009; Sokolova & Lebedinsky, 2013 and references therein). So far, only a few
245 hyperthermophilic carboxydotrophic isolates that can grow at temperature >80 °C have
246 been reported, and they are archaeal species of the genera *Thermococcus* and
247 *Archeoglobus* from marine hydrothermal environments (Sokolova *et al*., 2004; Henstra
248 *et al*., 2007; Lee *et al*., 2008). We observed potential microbial CO-oxidation in sample
249 UN-90 incubated at 90 °C. Previously, microbial CO oxidation has been reported in hot
250 springs as high as 80 °C and 90 °C (Kochetkova *et al*., 2011). These results suggest the
251 existence of unknown hyperthermophilic CO-oxidizers in terrestrial hot springs.
252 Significant potential microbial CO-oxidation activities were not observed in
253 the other hot spring samples, such as KS-65 and BZ-65, in which temperature and pH
254 conditions seemed suitable for CO-oxidizers. To investigate the differences between
255 positive and negative samples, principal component analysis (PCA) was carried out

using environmental parameters as shown in Fig. 3. Sample YG-65 was excluded from this analysis because an ORP value was not available. There were no apparent similarities among samples where potential microbial CO-oxidation activities were observed, though they were quite distinct from negative samples (Fig. 3). There was a rough tendency of positive samples to be relatively rich in nutrients (TOC and TN) and have higher ORP values and soil-water contents than negative samples. Among samples associated with the environmental temperature of approximately 65–71 °C and circum-neutral pH, the TOC and TN values of JI-65 and JI-70 (positive samples) were more than double those of KS-65 and BZ-65 (negative samples). ORP values of positive samples ranged from −49 mV to 487 mV and were higher than those of negative samples (Table 1). The OPR values were in a range of ‘reduced’ to ‘moderately reduced’ redox condition that appears too oxidative for sulphate reduction and methanogenesis (DeLaune & Reddy, 2005; Klüpfel *et al*., 2014). One of the presumed sources of CO in environments is methanogens and sulphate reducers because some of them produce CO under laboratory conditions (Conrad & Thauer, 1983; Lupton *et al*., 1984; Techtmann *et al*., 2009). However, in our case, it appeared that the metabolic activity of those microorganisms did not necessarily correlate with the distribution of CO-oxidizing anaerobes. Alternatively, CO is produced from volcanic gas and organic substances (e.g., plant matter and humus). CO production from organic matter is enhanced in the presence of oxygen, higher temperatures and higher water contents (Conrad & Seller 1985; Tarr *et al*., 1995; Shade *et al*., 1999; Hellebrand & Shade, 2008). Our result implies that the CO source is likely from organic substances and suggests that

278 CO-oxidizers prefer environments where high CO productivity can be expected (high
279 ORP values, TOC, TN and soil-water contents).
280
281 **Abundance of *Carboxydothermus* CODH-II genes and 16S rRNA genes**
282 Results of qPCR are shown in Fig. 4. The technical lower detection limit of qPCR for
283 the standard bacterial 16S rRNA gene, archaeal 16S rRNA gene and *cooS-II* were
284 1.0×10^{3}, 1.0×10^{3}, and 5.0×10^{1} copies/μL, respectively. In environmental samples,
285 *Carboxydothermus cooS-II* genes were detected in more than two replicates of samples
286 of UN-55, UN-90 and YG-65. *Carboxydothermus cooS-II* was also detected in one or
287 two replicates from UN-20, U-Lake, BZ-65, ME-90, JI-70 and JI-65. The highest copy
288 number was 6.05×10^{4} copies/g sediment in UN-55 corresponding to 7.95×10^{-4}% of
289 bacterial 16S rRNA. *Carboxydothermus cooS-II* copies were detected from samples
290 UN-90 and ME-90, where the environmental temperatures were above growth
291 temperature (≥78 °C) of *Carboxydothermus* spp. (Yoneda *et al*., 2012). On the other
292 hand, *Carboxydothermus cooS-II* was below detection level in sample KS-65 despite its
293 environmental temperature being optimal (65 °C) to most *Carboxydothermus* spp.
294 The qPCR series was conducted on CO-incubated samples used for
295 measurement of potential microbial CO-oxidation activities after incubation for a week
296 from the start time of the experiment. *Carboxydothermus cooS-II* was detected in more
297 than two replicates in CO-incubated samples UN-20, UN-55, UN-90, KS-90 and KS-65.
298 Interestingly, *Carboxydothermus cooS-II* was detected in CO-incubated KS-90 and
299 KS-65 samples where its corresponding environmental samples produced negative

results. In contrast, *Carboxydothermus cooS-II* was not detected in CO-incubated JI-70 and JI-65 samples. Abundances of *cooS-II* in environments and in CO incubated samples from UN-55 and UN-90 were compared by t-test. There were no significant differences in *cooS-II* copy numbers in either sample (Fig. 4).

Some of the representatives of qPCR products from environmental samples and CO incubated sediments were sequenced and the inner region of the *cooS-II* primers (123 bp) were compared to *cooS-II* of *C. hydrogenoformans* (CP000141), *C. ferrireducens* (IMG accession no. 2510417609) and *C. pertinax* (AB723512) obtained from DDBJ/EMBL/GenBank or Integrated Microbial Genome (IMG) databases (data not shown). A total of 58 sequences were obtained from the environmental samples. Of these, 31 sequences showed 97–100% similarity to *C. pertinax cooS-II*: UN-20 (4), UN-55 (8), UN-90 (6), U-Lake (3), YG-65 (3), BZ-65 (3), MI-90 (1) and JI-65 (3). A total of 23 sequences showed 98–100% similarity to *C. hydrogenoformans cooS-II* from samples UN-90 (7), U-Lake (1), YG-65 (2), BZ-65 (1), MI-90 (7), JI-70 (4). The remaining four sequences, all from UN-90, showed 94% similarity to both *Carboxydothermus ferrireducens* and *C. pertinax cooS-II*. A total of 66 sequences were obtained from CO incubated sediment samples. Of these, 39 sequences were most related to *C. pertinax cooS-II* with 97–100% sequence similarity: U-Lake (5), UN-20 (6), UN-55 (10), UN-90 (5), YG-65 (1), BZ-65 (2), KS-65 (5) and KS-90 (5). The other 27 sequences were most related to *C. hydrogenoformans* with 97–99% similarity: UN-20 (2), UN-55 (2), UN-90 (3), YG-65 (3), BZ-65 (5), KS-65 (8) and KS-90(4). The divergent sequences found in our study sites showed there are variations in

322 *Carboxydothermus* spp. and suggested that species-level diverse CO-oxidizers co-exist
323 in certain environments.
324
325 **Discussion**
326 To the best of our knowledge, our work is the first attempt to discover the distribution
327 and abundance of carboxydotrophic anaerobic thermophiles in various hydrothermal
328 environments in relation to environmental factors. Most carboxydotrophic anaerobic
329 thermophiles require strictly anaerobic, reduced conditions for carboxydotrophic growth.
330 However, most of them are not obligate carboxydotrophs and are able to utilize organic
331 substrate and electron acceptors such as ferric iron, AQDS
332 (anthraquione-2,6-disulfonate) and sulphur compounds (Sokolova *et al.*, 2009;
333 Sokolova & Lebedinsky, 2013). Those alternative metabolic pathways may enable
334 carboxydotrophic bacteria to survive in moderately reduced conditions. We observed
335 potential microbial CO-oxidation in samples where ORP ranged from −49 mV to 487
336 mV. Within this ORP range, respiration of iron, fumarate and humic substances can
337 take place (Klüpfel *et al.*, 2014), and CO-oxidation may take place as a subsidiary
338 reaction. Our results suggested that anaerobic CO-oxidizing activity is associated with
339 redox conditions, soil-water content, TOC and TN. TOC can be presumed as a CO
340 source where a positive association is conceivable between CO-oxidizing activity and
341 TOC value. Fig. 3 shows that TN is also a putative factor that influences potential
342 microbial CO-oxidation activity. Some CO-oxidizing thermophiles can reduce nitrates
343 as electron acceptors when grown on organic compounds such as yeast extract (e.g.,

Moorella thermoacetica) and lactate (e.g., *C. hydrogenoformans*) (Fröstl *et al*., 1996; Henstra *et al*., 2004). Further studies are needed to determine which nitrogen compounds are associated with the distribution or activity of CO-oxidizers. In environments rich in organic matter, the ability to withstand and utilize CO may be an advantage, allowing microbes to compete with fast-growing aerobes, as CO generally inhibits cytochrome oxidase within aerobic respiration (Cooper & Brown, 2008). Potential microbial CO-oxidation activities were detected in relatively high ORP sites, suggesting that CO-oxidizers may have a niche in an intermediate zone of aerobic and anaerobic environments thanks to their utilization of electron acceptors such as nitrate, Fe(III) and fumarate.

The abundance of *Carboxydothermus* spp. did not correlate with potential CO-oxidation activities and there were no apparent similarities in the environmental features among samples in which *Carboxydothermus* spp. were detected (Fig. 2, Fig. 3 and Fig. 4a). Moreover, no significant changes in the *Carboxdothermus* spp. population were observed between environmental samples and CO-incubated samples from UN-55 and UN-90 (Fig. 4a and 4b). In samples JI-65 and JI-70, abundance of *Carboxydothermus* spp. dropped below the threshold for detection despite the presence of potential microbial CO oxidation activity. These results indicate that there were other unknown CO-oxidizers responsible for CO-oxidation in some of our samples. Interestingly, the abundance of *Carboxydothermus* spp. was maintained or even increased to a detectable level when incubated at a high temperature of 90 °C (in sample UN-90 and KS-90), although sharp decreases in bacterial population were observed. *C.*

366 *hydrogenoformans* can produce spores (Wu *et al*., 2005) that may allow this species to
367 maintain its population in inhospitable environments. *Carboxydothermus* spp. can be
368 regarded as rare and robust bacteria that are distributed in a wide range of environments
369 including extremely thermophilic hot springs, non-hydrothermal environments, and
370 coastal environments.
371 Anaerobic CODH genes are widely distributed in physiologically diverse bacteria and
372 archaea. Their phylogenetic relationships are often incongruent with taxonomy based on
373 16S rRNA genes, especially clade F including *cooS-I*, *cooS-II* and *cooS-IV* of *C.*
374 *hydrogenoformans* (Techtmann *et al*. 2012). For example, a gene cluster which include
375 *cooS-I* in *C. hydrogenoformans* is closely related with that of *Thermosinus*
376 *carboxydivorans* indicating horizontal gene transfer of the cluster (Techtmann *et al.*
377 2012). Thus, we could not exclude the possibility of unintended detection of the
378 recipient microorganisms using our *cooS-II* primer set.
379 Because CO is a common trace chemical in environments, further study on diversity,
380 competition or habitat segregation would be of interest. In addition, further research
381 should include exploration of new habitats (e.g., extremely hot or acidic terrestrial hot
382 springs) for CO-oxidizing anaerobes.
383

384 **Acknowledgement**

385 We would like to thank Makoto Shibata for support in analysing the chemical properties
386 of the samples. We would like thank Dr. Stephen M. Techtmann for kindly providing
387 CODH gene sequence information that greatly improved our knowledge regarding

388 CODH and anaerobic carboxydotrophic microbes. We are also grateful to the local
389 people and the owners of the hot springs for willingly supporting our sampling.
390 This work was supported by a Grant-in-Aid for Scientific Research (A) (No.
391 20248023) and (A) (25252038) from The Ministry of Education, Culture, Sports,
392 Science and Technology (MEXT) and a Grant-in-Aid for JSPS Fellows (No. 244441)
393 from the Japan Society for the Promotion of Science (JSPS).

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Figure legends

Fig. 1. Sampling sites in (a) the Kagoshima Pref. in southern Kyushu Island and (b) Shizuoka Pref.

Original maps were obtained from a digital map provided by The Geospatial Information Authority of Japan (GSI) (http://www.gsi.go.jp/) and have been edited by the authors.

Fig. 2. Potential microbial CO-oxidation in environmental samples

Each result of regression analysis is shown outside each graph. Lines showing potential microbial CO-oxidation are dotted when negative in the regression analysis.

Fig. 3. Principal component analysis of environmental features

Closed symbols show positive samples for potential microbial CO-oxidation.

Fig. 4. Abundance of the *Carboxydothermus cooS-II* and 16S rRNA genes in (a) environmental samples and (b) in CO-incubated samples

CooS-II bars are average values of positive replicates (negative replicates are not included in the analysis). Error bars are shown when the genes were detected in more than two replicates (i.e., detected at least in triplicate).

Fig. 1

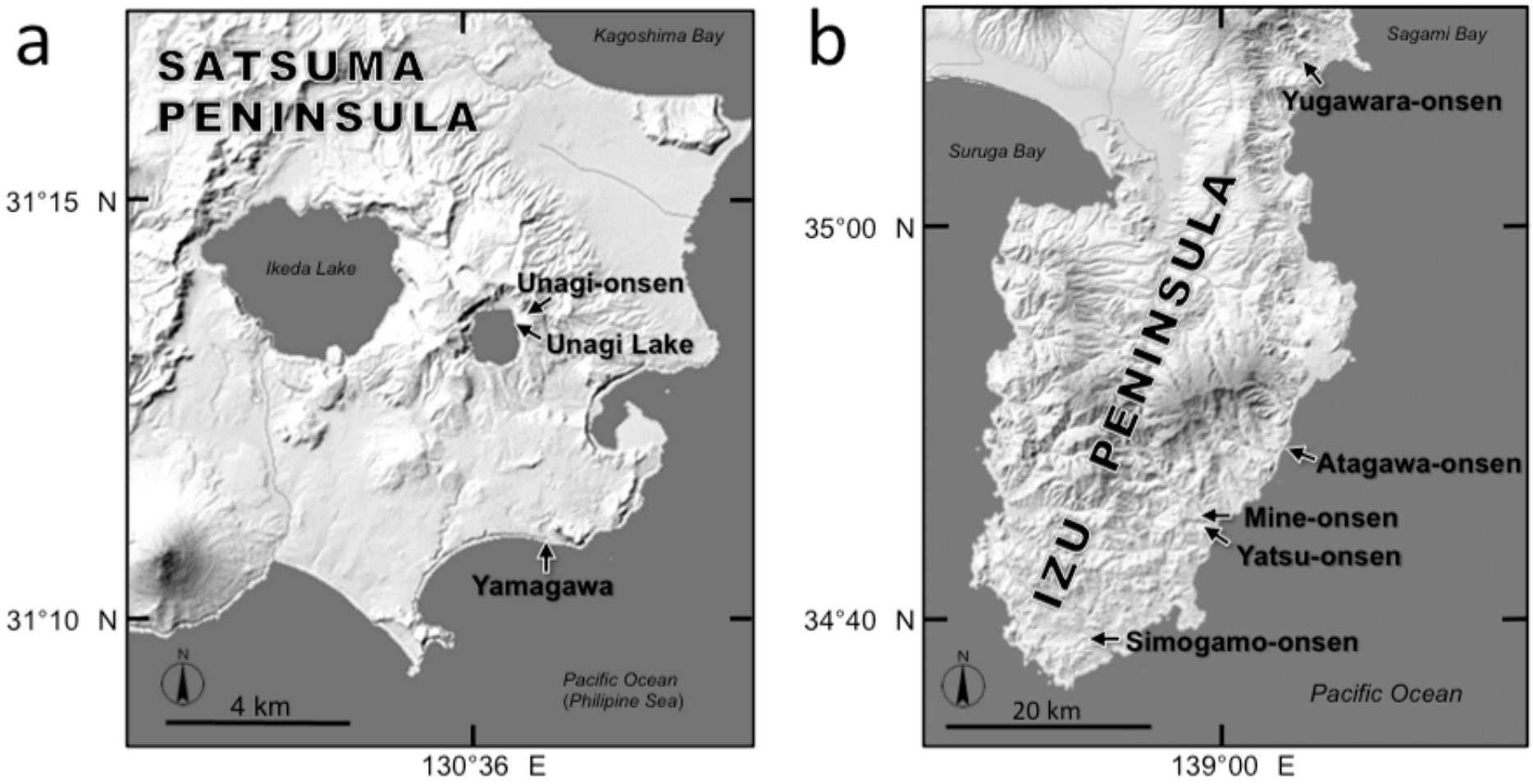
a
SATSUMA
PENINSULA
Kagoshima Bay
Ikeda Lake
Unagi-onsen
Unagi Lake
Yamagawa
Pacific Ocean
(Philipine Sea)
4 km
N
31°15 N
31°10 N
130°36 E
b
Sagami Bay
Yugawara-onsen
Suruga Bay
IZU PENINSULA
Atagawa-onsen
Mine-onsen
Yatsu-onsen
Simogamo-onsen
Pacific Ocean
20 km
N
35°00 N
34°40 N
139°00 E

Fig. 2.

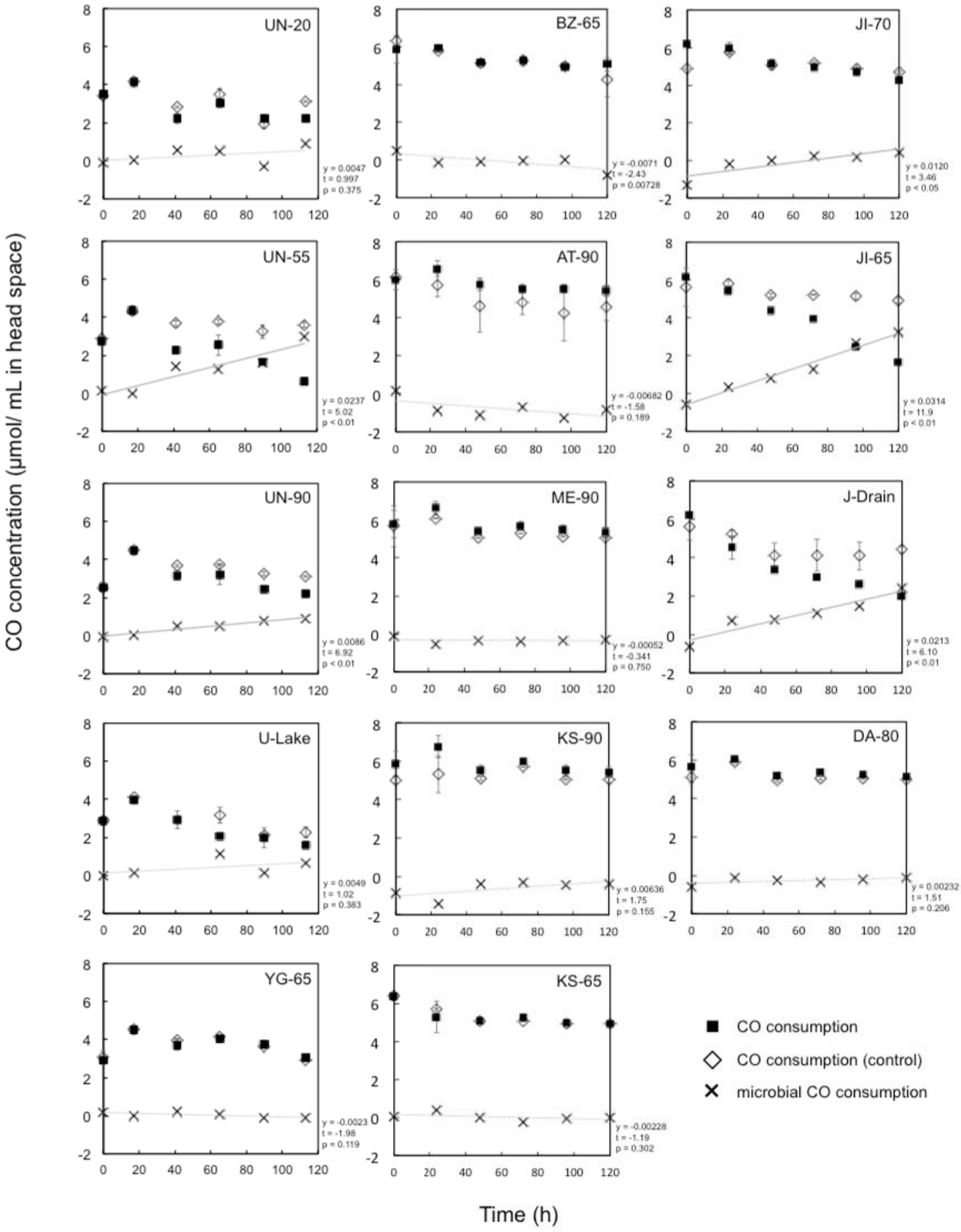

Fig. 3

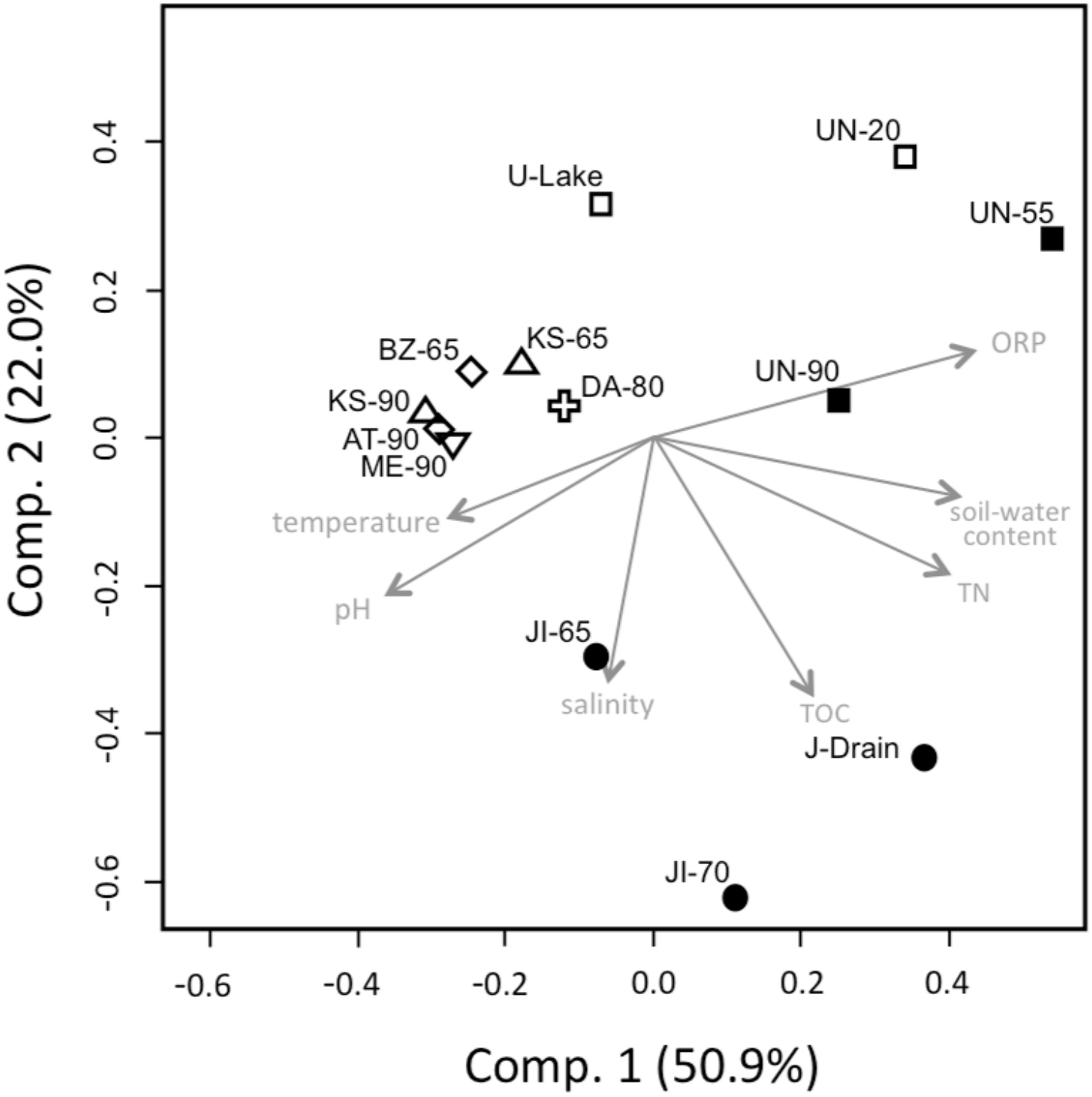
UN-20
U-Lake
UN-55
BZ-65
KS-65
ORP
UN-90
DA-80
KS-90
AT-90
ME-90
temperature
soil-water content
TN
pH
JI-65
salinity
TOC
J-Drain
JI-70
Comp. 2 (22.0%)
Comp. 1 (50.9%)
0.4
0.2
0.0
-0.2
-0.4
-0.6
-0.6
-0.4
-0.2
0.0
0.2
0.4

Fig. 4